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Cellular oxidative damage is more sensitive to biosynthetic rate than to metabolic rate: A test of the theoretical model on hornworms (*Manduca sexta* larvae)

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ABSTRACT

We develop a theoretical model from an energetic viewpoint for unraveling the entangled effects of metabolic and biosynthetic rates on oxidative cellular damage accumulation during animal's growth, and test the model by experiments in hornworms. The theoretical consideration suggests that most of the cellular damages caused by the oxidative metabolism can be repaired by the efficient maintenance mechanisms, if the energy required by repair is unlimited. However, during growth a considerable amount of energy is allocated to the biosynthesis, which entails tradeoffs with the requirements of repair. Thus, the model predicts that cellular damage is more influenced by the biosynthetic rate than the metabolic rate. To test the prediction, we induced broad variations in metabolic and biosynthetic rates in hornworms, and assayed the lipid peroxidation and protein carbonyl. We found that the increase in the cellular damage was mainly caused by the increase in biosynthetic rate, and the variations in metabolic rate had negligible effect. The oxidative stress hypothesis of aging suggests that high metabolism leads to high cellular damage and short lifespan. However, some empirical studies showed that varying biosynthetic rate, rather than metabolic rate, changes animal's lifespan. The conflicts between the empirical evidence and the hypothesis are reconciled by this study.

1. Introduction

The deleterious products of oxidative metabolism, such as reactive oxygen species, cause various forms of cellular damages, which in turn undermine the organism's health maintenance and longevity (Balaban et al., 2005; Barja, 2004). To counteract the accumulation of damage, organisms have evolved highly efficient repair mechanisms, such as oxidant scavenging and damage repair (Beckman and Ames, 1998; Monaghan et al., 2009). These repair mechanisms require energy and resources. If the resources and energy that could be allocated to repair are otherwise channeled to other biological processes, then damage will inevitably accumulate despite the high repair efficiency (Monaghan et al., 2009).

Biosynthesis during growth, one of the most intensively investigated biological processes that trades off with repair, is positively correlated with oxidative damage level and proxies of it, such as declined performance and shortened lifespan at whole organismal level (Hou, 2013; Mangel and Munch, 2005; Mangel and Stamps, 2001; Miller et al., 2002) and molecular and cellular level (Bartke, 2005; Rollo et al., 1996). Rapid growth leads to higher phospholipid peroxidation (Nussey et al., 2009), protein carbonyl content (Forster et al., 2000), decreased antioxidant defenses in red blood cells (Alonso-Alvarez et al., 2007), elevated free radical processes (Rollo et al., 1996), declined locomotion ability (Mangel and Stamps, 2001), impaired immune function (De Block and Stoks, 2008), and higher mortality rate and shortened lifespan (Bartke, 2005; Merry, 1995; Miller et al., 2002). A special type of rapid growth—catch up growth, referring to infants with low birth weight reaching to or exceeding the normal body weight later in life, increases the risk of adult-onset metabolic syndromes and short lifespan in human and laboratory rodents (Metcalfe and Monaghan, 2001). In contrast, suppressed growth, usually induced by food restriction (Masoro, 2005; Merry, 2002; Weindruch and Walford, 1988) or genetic modification of growth hormone (Bartke, 2005; Brown-Borg, 2003), has been long known to keep animals in a relatively youthful and healthy state, and greatly extends lifespan in a broad diverse of species, indicating the up-regulations of somatic damage repair in these animals. We need to emphasize that the observed relationship between biosynthesis and cellular damage and/or longevity is not merely a statistical

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correlation. As many studies have shown (Hou, 2013; Hou and Amunugama, 2015; Lee et al., 2011; Lee et al., 2013; Mangel and Munch, 2005; Metcalfe and Monaghan, 2003), the nature of this association is causal and effect. We will give detailed explanations in the following sections and the Supplementary Materials.

Attempting to interpret the positive correlation between biosynthesis and cellular damage, many researchers have argued that increased biosynthetic rates cause increased metabolic rate, which, as a primary source of free radicals, in turn leads to increased cellular damage (Monaghan et al., 2009; Nussey et al., 2009). However, although biosynthesis is fueled by metabolism, the relationship between them is not simply proportional. When one of them increases, the other may increase (Ricklefs, 2003; West et al., 2001), decrease (Hayes et al., 2015; Steyermark, 2002), or keep roughly the same (Álvarez and Nicieza, 2005; McCarter and Palmer, 1992). The complex relationships between them make their effects on cellular damage difficult to isolate. Rates of metabolism and biosynthesis may have different degrees of impacts on cellular damage, i.e., the same degree of variations in these rates may lead to different relative changes in damage. However, in most studies the observed changes in cellular damage reflect the combined influences of changes in both metabolic and biosynthetic rates. When these two rates vary independently or even in the opposite direction, the separate effects of each on cellular damage are obscured.

The goal of this paper is to unravel the effects of biosynthetic and metabolic rate on cellular damage accumulation. We first develop a simple theoretical model based on the first principle of energy tradeoffs and real physiological parameters. The model predicts that, if the repair efficiency is high, then the changes in damage level caused by the changes in metabolic rate is negligible, and the damage level is more sensitive to the changes in biosynthetic rate. We then test the model by experiments on the 5th instar tobacco hornworms (the last instar of *Manduca sexta* larvae). We measured lipid peroxidation and protein carbonyl as two indexes of cellular damage accumulation in larvae with different rates of growth and metabolism.

2. Methods

2.1. The theoretical model

Recently we have developed a theoretical model grounded on empirical data for understanding how animals alter their energy budgets on damage repair, biosynthesis, and energy storage in the face of environmental changes, and how the alteration in energy budget affects the cellular damage accumulation (Hou, 2013; Hou, 2014; Hou and Amunugama, 2015; Hou et al., 2011). Some of the quantitative predictions on relationship between growth suppression and lifespan extension are strongly supported by data collected from wild animals across a broad range of species and >200 empirical studies on small laboratory rodents (Hou, 2013; Hou and Amunugama, 2015; Hou et al., 2011).

In this paper, we extend the model and make predictions on the relationship between cellular damage, metabolic rate, and biosynthetic rate. The detailed assumptions and derivation of the equations of the model have been published (Hou, 2013; Hou, 2014; Hou and Amunugama, 2015). Here we only introduce it briefly. The key idea of the model is summarized in Fig. 1. In brief, the oxidative metabolism causes cellular damage. Animals have highly efficient repair mechanisms, which require energy. If the "repair engine" has adequate energy, then most of the damage can be repaired, regardless of how high the oxygen consumption rate is (as shown in Fig. 1(A)). However, during growth, animals allocate a considerable amount of metabolic energy to biosynthesis, so the energy allocated to repair is inadequate. Although the "repair engine" is still efficient, the net damage will be large due to the low energy input (as shown in Fig. 1(B)).

The theoretical model is based on two assumptions.

Assumption 1. Within a species, the rate of somatic damage production, H, caused by deleterious products of oxidative metabolism, such as reactive oxygen species (ROS), is proportional to the rate of oxygen consumption (metabolic rate, B), as $H = \delta B$, where δ is a constant within a species, indicating the amount of damaged mass associated with one unit of metabolic energy. The proportionality between ROS production and metabolic rate holds generally within one species living under normal conditions. However, under certain conditions, ROS production can be disproportionally lower for a given metabolic rate (lower δ). We discuss in detail how our model handles these cases in the section of Discussion.

Assumption 2. Repairing the damage requires metabolic energy. The rate of repair, R (repaired mass/time), is proportional to the energy available for maintenance, B_{maint}, with a coefficient η, i.e., $R = \eta B_{maint}$, where η is also a constant, indicating the amount of mass that can be repaired by one unit of available metabolic energy. The energy allocated to maintenance is the difference between the total metabolic energy (B) and the energy spent on biosynthesis B_{syn}, i.e., $B_{maint} = B - B_{syn}$ (West et al., 2001).

The net damage, $H - R$, accumulates, which can be integrated as a function of time, i.e., $F(t) = \int_0^t (\delta B - \eta B_{maint}) d\tau$. We rewrite this equation as $D(t) = F(t)/\delta = \int_0^t (B - \varepsilon B_{main}) d\tau$, where $\varepsilon = \eta/\delta$ is the effective repair efficiency, indicating the ratio of repaired mass and damaged mass for one unit of energy, and $D(t)$ can be considered the recalibrated net cellular damage. To estimate damage, we substitute the equations $B = B_{maint} + B_{syn}$ in $D(t)$, and obtain

$$\begin{aligned} D(t) &= \int_0^t (B - \varepsilon \times B_{maint}) d\tau \\ &= \int_0^t B - \varepsilon \times (B - B_{syn})] d\tau \\ &= (1-\varepsilon) \int_0^t B d\tau + \varepsilon \int_0^t B_{syn} d\tau \\ &= (1-\varepsilon) \times ME + \varepsilon \times SE \end{aligned} \quad (1)$$

where $ME = \int_0^t B d\tau$ is the metabolic energy spent during growth (in unit of joules); $SE = \int_0^t B_{syn} d\tau$ is the energy spent on biosynthesis during

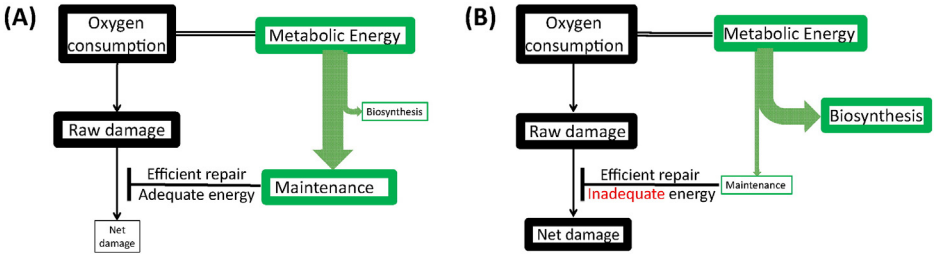

Fig. 1. Schematic illustration of the tradeoff between maintenance and biosynthesis. In Panel (A) and (B), the hypothetical animals have the same level of oxygen consumption (metabolic rate), and the same level of raw cellular damage. The animal in Panel (A) allocates small amount of energy to biosynthesis so that the efficient repair mechanism acquires adequate energy, and the net damage level is low. In contrast, the animal in Panel (B) allocates a large fraction of metabolic energy to biosynthesis. Thus, despite the high repairing efficiency the net damage level is high due to inadequate energy allocation to maintenance.

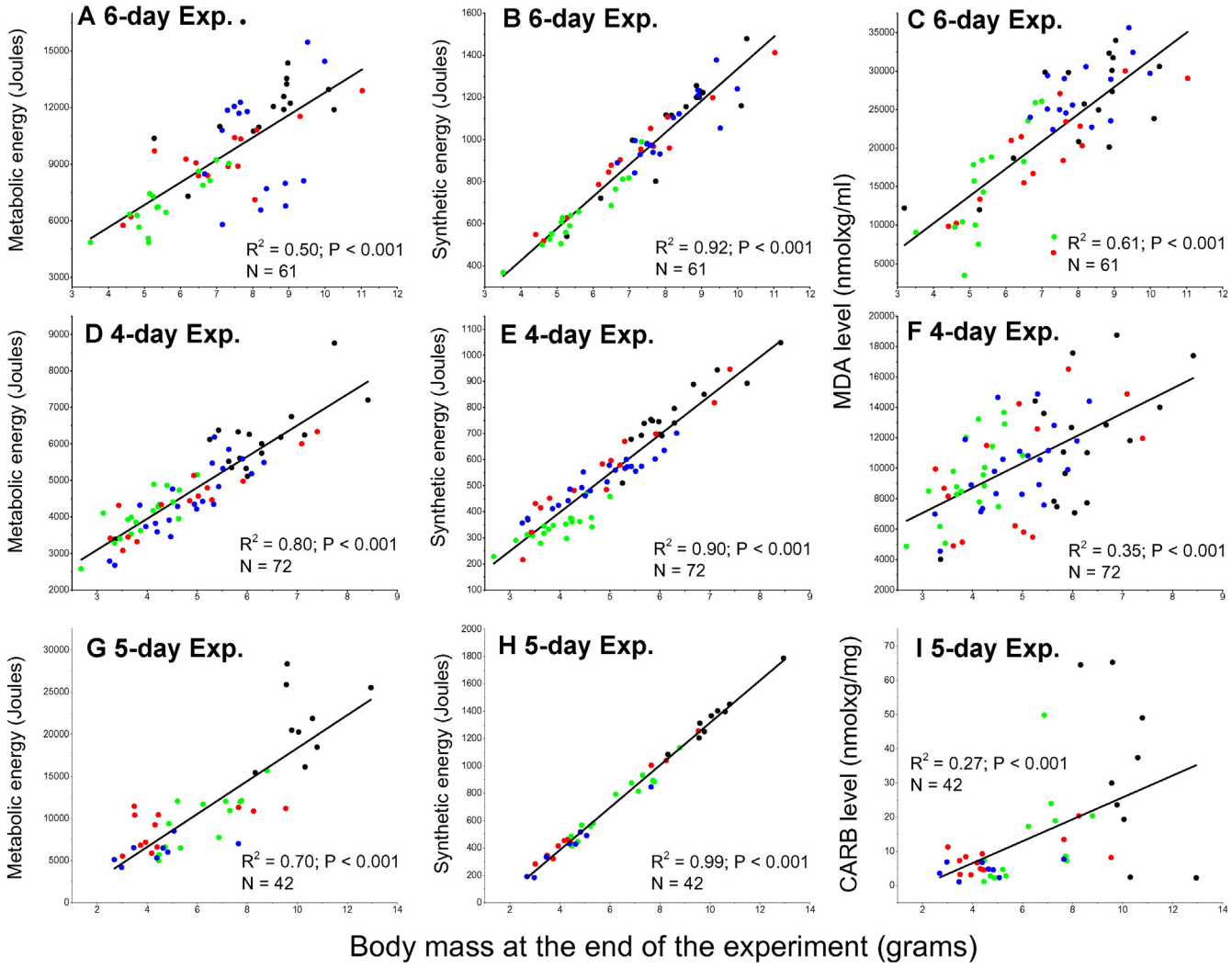

Fig. 2. Food treatments induce 4-fold of variation in metabolic energy (Panel (A), (D), and (G)), 4-fold of variations in synthetic energy (Panel (B), (E), and (H)), and 9-fold of variation in damage level (Panel (C), (F) and (I)), respectively. Each dot represents an individual caterpillar that belongs to a food treatment cohort. Four cohorts represented in colors are: black: AL, red: SFR-A, blue: SFR-B, and green: LFR. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

growth, which is the product of the increase of body mass and the energy required to synthesize one unit of biomass.

Eq. (1) decomposes the net damage accumulation in two terms, $D_B = (1-\varepsilon) \times ME$ and $D_{syn} = \varepsilon \times SE$, estimating the effects of metabolism and biosynthesis on damage accumulation separately. Both terms are proportional to energy factors (*ME* and *SE*) with coefficients $1 - \varepsilon$ and ε respectively. The sensitivities of damage to the changes in metabolic and biosynthetic rate depend on the coefficients of these two terms, $1 - \varepsilon$ and ε. Based on fitting of empirical data and the first principle of biochemistry for cellular oxidation by ROS, the repair efficiency ε has been estimated to be in the neighbourhood of 0.99 ((Hou, 2013; Hou et al., 2011) and the Supplementary Material). For such a high efficiency, the metabolic term in Eq. (1), $(1-\varepsilon) \times ME$, is close to zero, regardless how metabolic energy (*ME*) changes. The major contribution to the net damage therefore comes from the biosynthetic term, $\varepsilon \times SE$. This means that the damage accumulation is more sensitive to the biosynthetic term (*SE*) than to the metabolic term (*ME*).

Table 1
Description of 6-, 5-, and 4-day food treatments.

	6-Day experiment		5-Day experiment		4-Day experiment	
Cohort	Day 1–3	Day 4 and 5	Day 1 and 2	Day 3 and 4	Day 1 and 2	Day 3
AL	AL	AL	AL	AL	AL	AL
SFR-A	AL	FR	AL	FR	AL	FR
SFR-B	FR	AL	FR	AL	FR	AL
LFR	FR	FR	FR	FR	FR	FR

In each experiment, we applied different food treatments to four cohorts, free-feeding (AL), Short-food-restriction-A (SFR-A), Short-food-restriction-B (SFR-B), and Long-food-restriction (LFR). Table 1 describes the length of each kind of food treatment. For example, in the 6-day experiment, for the cohort SFR-A, the food supply was ad libitum (AL) from day 1 to day 3, and then switched to food restriction (FR) from Day 4 to Day 5. For larvae under food restriction, the amount of food was given based on individual's body mass, as $F = 0.27 \times m + 0.44$, where F and m are the wet mass of food and body in unit of gram. This restriction level is designed based on our previous result (Hayes et al., 2015), so that the food uptake rate of food restricted cohorts is about 50% of that of the AL cohort.

Here, we test whether cellular damage is more sensitive to growth rate than to metabolic rate, as predicted by our theoretical model. We induced broad variations in metabolic energy (*ME*) and synthetic energy (*SE*) among individual 5th instar hornworms by varying the level of food supply in three experiments, namely 4-day, 5-day and 6-day food treatment. We then assayed the corresponding protein and lipid damage levels in individual hornworms, using protein carbonyl from midgut tissue (CARB) and plasma malondialdehyde (MDA) as surrogates. The carbonyl groups are produced on protein side chains when they are oxidised (Dalle-Donne et al., 2003) and the protein carbonyls are accumulated (Negre-Salvayre et al., 2008). MDA is one of the secondary decomposition products of polyunsaturated fatty acids generated by lipid peroxidation, and is widely used to assess oxidative damage level (Hall

et al., 2010; Monaghan et al., 2009; Nussey et al., 2009). MDA makes adducts with proteins and induces cell dysfunctions that are responsible for disrupting normal cell responses. MDA and other aldehydes formed during lipid peroxidation make carbonyl compounds with proteins and these compounds are found to be accumulated during aging. MDA adducts form fluorescence pigment lipofuscin, and it is progressively accumulated with aging (Negre-Salvayre et al., 2008), and MDA-lysine adduct is accumulated in plasma proteins and in hepatocytes in iron overloaded rats (Houglum et al., 1990).

We assume that the levels of MDA and CARB are proportional to the total cellular damage (variable, D, in Eq. (1)) with a factor g, asMDA (or CARB) $= g \times D$, and therefore Eq. (1) becomes

$$Damage = g \times (1-\varepsilon) \times ME + g \times \varepsilon \times SE, \quad (2)$$

where *Damage* denotes the level of MDA or CARB.

We need to emphasize that damage accumulates over the entire growth, so a considerable fraction of damage assayed in this study was accumulated during the first four instars of the larval lives, whereas our treatments only started when the larvae entered the 5th instar. Thus, to test how variations in these rates influence the damage accumulation, we must remove the effects of un-manipulated *ME* and *SE* in the first four instars from the assayed damage level (MDA and CARB). Previous studies, as well as this study, show that both *ME* and *SE*, the metabolic and synthetic energy spent during a period of growth, are linearly proportional to the body mass at the end of this period (see Fig. 2A, B, and (West et al., 2001)). So, removing the effects of *ME* and *SE* during the first four instar can be done by removing the effect of the body mass at the end of the 4th instar from assayed damage. We linearly regressed the damage level on this body mass. The residual of damage after the removal of this mass is then considered the damage caused by *SE* and *ME* during the 5th instar period—the experimental period. The damage level during the 5th instar, *SE*, and *ME* are all linearly correlated to the final body mass at the end of the experimental period, *M* (Fig. 2). This means that the final body mass has a confounding effect when performing a linear regression of the damage level during the 5th instar on *ME* and *SE*. We investigate the confounding effect of final mass on these variables in two ways. First, we control the final mass by including it in a multiple linear regression analysis with *SE* and *ME* to predict *Damage* level at 5th instar (details are described in Methods section). We also run an alternative model by removing the confounding effect of final mass by performing separate linear regressions of *Damage*, *ME*, and *SE* on final body mass and calculating the mass residuals in each of the three analyses. We then regress the mass residual of *Damage* on the mass residuals of *ME* and *SE*, as

$$Damage_{\text{residual}} = \beta_0 + \beta_1 \times ME_{\text{residual}} + \beta_2 \times SE_{\text{residual}} \quad (3)$$

where β_0, β_1, and β_2 are estimated regression coefficients. Eq. (3) allows us to write the model in the form of Eq. (2) for comparison. We present results from both approaches, but use the model employed for regression Eq. (3) as the focus of our predictions.

Comparing the theoretical Eq. (2) and the regression Eq. (3), we make three predictions.

First, the fitted regression coefficient of the metabolic term, β_1, is smaller than that of the biosynthetic term, β_2;

Second, the ratio of the coefficients, β_1 and β_2, gives $\beta_1/\beta_2 = (1-\varepsilon)/\varepsilon$. We predict that the repairing efficiency (ε) estimated from this equation is in the neighbourhood of 0.99, which is the value previously estimated from data fitting and biochemistry principles for cellular oxidation by ROS. (Hou, 2013; Hou et al., 2011) (Details of estimating ε are given in the Supplementary Material).

And third, and more importantly, the partial correlation between *Damage* level and the metabolic term (*ME*) is insignificant after accounting for *SE* (*P*-value > 0.05), whereas that between *Damage* and the synthetic term (*SE*) is significant after accounting for *ME* ($P < 0.05$);

2.2. Animal rear and food supply levels

We induced variations in metabolic energy (*ME*) and biosynthetic energy (*SE*) by varying food supply levels in 4-day food treatment and 6-day treatment for MDA measurements, each with approximately 80 hornworms. Another ~40 hornworms were treated for 5 days for protein carbonyl measurements. The details of animal rearing are available in the Supplementary Material. In short, on the first day of the 5th instar, larvae were randomly separated and treated with four levels of food supply. The four cohorts were free-feeding (AL), short-term food restriction-A (SFR-A), short-term food restriction-B (SFR-B), and long-term food restriction (LFR). The length of free feeding and food restriction and the level of food restriction for each cohort are described in

Table 2
Linear regression results of *Damage* level (both MDA and CARB) on metabolic energy (*ME*) and biosynthetic energy (*SE*), using Model B.

Model B without measurement error: $Damage_{\text{residual}} = \beta_0 + \beta_1 \times ME_{\text{residual}} + \beta_2 \times SE_{\text{residual}}$

	Experiment	Coefficients	*t*-Values	*P*-values	Partial correlation	VIF
β_0	6-day MDA	1.22×10^{-11}	0.000	1.000	_	_
	5-day CARB	-1.22×10^{-14}	0.000	1.000	_	_
	4-day MDA	2.5×10^{-8}	0.000	1.000	_	_
β_1 of ME_{residual}	6-day MDA	0.372	1.060	0.294	0.138	1.20
	5-day CARB	0.001	1.688	0.099	0.261	1.07
	4-day MDA	0.394	0.651	0.517	0.078	1.11
$\beta_2$of SE_{residual}	6-day MDA	32.608	3.384	0.001	0.406	1.20
	5-day CARB	0.109	2.146	0.038	0.325	1.07
	4-day MDA	9.949	1.913	0.060	0.224	1.11

$R = 0.408$ (6-day), $= 0.499$ (5-day), and $= 0.50$ (4-day)
Condition index $= 1.54$ (6-day), $= 1.29$ (5-day), and $= 1.38$ (4-day)
$N = 72$ (4-day), $= 42$ (5-day), and $= 61$ (6-day)

Model C with measurement error (6-day experiment)

	Coefficients	*t*-Values	*P*-values	Partial correlation	VIF
β_0	-1.64×10^{-11}	0.000	1.000	_	_
β_1 of ME_{residual}	0.386	1.09	0.285	0.138	1.20
$\beta_2$of SE_{residual}	32.762	3.44	0.001	0.406	1.20

$R = 0.408$; Condition index $= 1.54$

In Table 2, we list the results of the multiple linear regression using Model B. For each coefficient, namelyβ_0,β_1, andβ_2, we show the results from the 4-, 5-, and 6-day experiments. At the bottom of the table, we list the R values and condition indexes of the overall regression for each data-set. The parameter, E_m, was taken to be 177 J/g for the regression.

Table 1. All larvae were sacrificed at the end of the 6-day and 4-day experiments for MDA and 5-day experiment for CARB measurements.

2.3. Synthetic energy spent during the experimental period

Body mass of each larva in every cohort was measured approximately at the same time every day from the first day of the 5th instar to the nearest 0.1 mg using a digital microbalance (Perkin-Elmer AD6). The energy spent on biosynthesis during the experiment, *SE*, in unit of Joules, was calculated as the increment of body mass from the 1st day to the last day of the experiment, Δm, multiplied by the energy required to synthesis one unit of biomass, E_m , i.e., $SE = \Delta m \times E_m$. The value of E_m in 5th instar hornworm was taken to be 177 J/g, which is very close to the value estimated by Sears et al., 168 J/g (Sears et al., 2012) (1197 J/g of dry mass, and dry/wet mass ratio is 14% throughout the 5th instar). Our independent assays using the method described in Peterson et al. (Peterson et al., 1999) gave a range of this parameter from 143 to 212 J/g (Details in the Supplementary Materials). We took the average, 177 J/g, and the upper and lower limit of the range estimated by us (143 and 212 J/g) to perform the data analysis.

2.4. Metabolic energy spent during the experimental period

The details of respirometry are available in the Supplementary Material and reference (Hayes et al., 2015). In short, we measured the exchange rate of O_2 and CO_2 of each larva for 7–10 min time interval every day using Sable System International (Las Vegas, U.S.A.) CA-10 CO_2 and FC-10 O_2 analyzers at 25 °C. We then converted them to metabolic rate (in Watts). We assumed that metabolic rate of each caterpillar increases linearly between two successive measurements (approximately 24 h apart) due to the body mass increase during the day. Based on this assumption, we calculated the metabolic energy consumed in a particular day as 24 h multiplied by the mean value of the rates measured at the beginning and the end of the 24-h period in unit of Joules. The metabolic energy (*ME*) was defined as the sum of larval metabolic energy expenditure each day during the experiment in unit of Joules.

Due to the random activity rhythm of hornworms, metabolic rate did not keep a constant, even after the effect of increasing body mass was removed. Thus, the random activity produced an inherent unknown measurement error. We incorporated the amount of variability in the measurement errors into the statistical analysis. Measurement errors were estimated for each individual as follows. The gas analyzers took samples every second, so the ~10 min of respirometry generated a time-series curve. We calculated the standard deviation of each curve, which was the estimated measurement error of metabolic rate of one individual caused by the random activity. We then calculated the percentage of the standard deviation as SD/mean × 100%, and assumed this percentage represents the random activity during the day. The standard deviation in the estimated measurement errors across individuals was then used as the estimated measurement error standard deviation in the ensuing statistical analysis. This approach to estimating measurement error variation is described in (Bland and Altman, 1996).

2.5. MDA and CARB assays

The MDA-HPLC method described by (Lin et al., 2006) was optimized. The details are available in the Supplementary Material. In short, we used HPLC with Alltima C18 column to assay the total plasma MDA (free plus protein-bound) from the hemolymph samples of larvae 4-day and 6-day in their final instar. The assay depends on the formation of adducts between MDA and thiobarbituric acid (TBA) under heat.

For the protein carbonyl assay, we modified the protocols (Krishnan and Kodrík, 2006; Levine et al., 1990), which are based on spectrophotometric measurement of 2,4-dinitrophenylhydrazine (DNPH) derivatives of protein carbonyls in midgut tissues of larvae 5-days in their final instar. The details are available in the Supplementary Materials.

The measured damage levels, MDA concentration (in unit of nM/mL) and protein carbonyl (in unit of nM/mg), are body mass-specific quantities, whereas our model (Eqs. 1–3) makes predictions on the total damage, metabolic energy, and biosynthetic energy in the whole body. Thus we multiplied the damage level by the larval body mass on the last day of the experiment, and used this value to test our theoretical model. To keep the dimensions the same in the equation, we could also use the per ml values of MDA and the per mg values of CARB, and divide *ME* and *SE* by body mass, so that all the variables are mass-specific. However, this would introduce the variable of body mass twice in the regression equation (to *ME* and *SE*), and would give less accurate results, compared to the method that only introduces body mass once (to damage). Moreover, the mass-specific (per mass) quantity can still be strongly correlated to body mass. There are many such examples in physiology (Hou et al., 2008; Kooijman, 2010). In our study, damage per ml or mg, mass-specific *ME*, and mass-specific *SE*, were all correlated to body mass ($R = 0.3$, $P < 0.02$ for damage, $R = 0.6$, $P < 10^{-6}$ for mass-specific *SE*, and $R = 0.24$, $P < 0.037$ for mass-specific *ME*). Thus, even if we take the mass-specific quantities to perform the multiple linear regression, we would still need to remove the confounding effect of body mass by estimating the mass-residuals of the variables.

2.6. Data analysis and statistics

In each experiment, we tested if food treatments induce significant difference in *Damage* level (MDA and CARB) between each cohort. We performed ANCOVA with *Damage* as the dependent variable, *ME* and *SE* as the covariates, and food treatment as the fixed factor, using SPSS 21. ANCOVA yielded $p > 0.1$ between each pair of these four treatments (see Results), indicating that the food treatments did not induce any difference in *Damage* level. Thus, in each experiment we pooled data from four cohorts and regressed *Damage* level on *ME* and *SE*. Note that data for each of the 4, 5, and 6 day experiments were analyzed separately due to the inherent differences in the experiments that were performed at different times. The 5 day experiment used a different damage assay, while the 6 day induced more variation in damage than 4 day perhaps because most of the body mass increase occured between the 3rd and 6th days.

However, this regression will cause two problems. First, these three variables were linearly proportional to the final body mass on the last day of the experiment. Thus, the confounding effect of body mass may give false correlations between the dependent and independent variables. Second, because of the confounding effects of body mass, *ME* and *SE* were correlated to each other. Thus, there may be multicollinearity between the independent variables, which often leads to unreliable and unstable estimates of the regression coefficients in multiple regression. When two independent variables are highly correlated, the one measured less accurately will usually fall out as non-significant.

To address these issues, we first fit a multiple linear regression model (Model A) with *ME*, *SE*, and final body mass (*M*) as predictors of $Damage_W$, the whole body *Damage* during the 5th instar. This model controled for the confounding effect of final body mass, but introduces severe multicollinearity due to the high correlation between final body mass and the other two predictors. In our second regression model (Model B), we removed the confounding effect of final mass on the variables by calculating the mass residuals of each variable, and then regress the mass residual of *Damage* on the mass residuals of *ME* and *SE*, as shown in Eq. (3).

Model A: $Damage_W = \beta_0 + \beta_1 \times ME + \beta_2 \times SE + \beta_3 \times M$.

Model B (Eq. (3)): $Damage_{residual} = \beta_0 + \beta_1 \times ME_{residual} + \beta_2 \times SE_{residual}$.

To make sure that the independent variables (the mass residuals of *ME* and *SE*) do not have multicollinearity in the multiple regression, we calculated the variance inflation factors (VIF) and condition index of the multiple regression. It has been commonly recommended that if the value of VIF is below 10, and condition index is below 30,

multicollinearity is not significant (Hair et al., 1995). We compared the estimates for β_1, β_2, and ε in all three models. These values should be most alike between models A and B since both are ways to adjust for the confounding effect of final mass.

We then included the measurement errors in the second model (Model B). A linear regression model ($Damage_i = \beta_0 + \beta_1 ME_i + \beta_2 SE_i + error_i$) was initially fit using *ME* and *SE* as explanatory variables to predict *Damage*. In the standard linear regression model, the explanatory variables are assumed to be measured without error, but this assumption is misleading since *ME* cannot be measured perfectly as we described above. Thus, a linear regression model that accounts for the measurement error in *ME* was fit using a latent variable approach (Fuller, 2009) in PROC CALIS, SAS v.9.4. In this model, $ME_i^* = ME_i + u_i$ where ME_i^* represents the observed *ME* value, ME_i represents the true (latent) value, and u_i represents the measurement error for individual *i*. It is assumed that the measurement error is independent from the true value. The size of the measurement error standard deviation (σ_u) is required to estimate the regression coefficients and is estimated as the following. We estimated the standard deviation of metabolic rate as the percentage of the mean value of each sample curve. This procedure is described in the previous section. This way, we obtained a distribution of the measurement errors (percentage values). We then estimated the standard deviation of the distribution, which is considered the size of the measurement error standard deviation (σ_u), and used to estimate the regression coefficients (Fuller, 2009).

3. Results

In the 6-day experiment, food treatments induced broad ranges of variation in metabolic energy (*ME* from 4850 J to 16,540 J), synthetic energy (*SE* from 370 J to 1480 J), and MDA level from 3510 nmol × g/ml to 35,610 nmol × g/ml (Fig. 2). All these variables were linearly proportional to the final body mass on the 6th day. The 4-day and 5-day experiments had the similar results (Fig. 2). Starting and ending body mass for all the treatments are given in the Supplementary Material (Table S1). The treatments did not induce any difference in *Damage* levels in either 4-day, 5-day, or 6-day experiment. ANCOVA, using *Damage* as the dependent variable, *ME* and *SE* as covariates, and food treatments as fixed factors, showed that the assumption of homogeneous regression slopes was satisfied ($P > 0.05$), and there was no difference in *Damage* level between each pair of these four treatments ($P > 0.1$, Fig.2).

3.1. Results from linear regression model A

For the 6-day MDA assay, statistical values of model A (Table S2) showed that *ME* and *M* were insignificantly correlated with MDA ($P > 0.05$), but *SE* was significant ($P < 0.005$). The 4-day MDA results showed insignificance for *ME*, *SE* and *M* ($P > 0.05$), but *SE* was at the edge of significance ($P = 0.062$). The 5-day CARB assays showed that *SE* and *M* were significantly correlated with CARB ($P < 0.05$), whereas M*E* was insignificant ($P > 0.05$). However, in all these three experiments, VIF values for *SE* and *M* were larger than 10 and condition indexes were larger than 30. So, we concluded the presence of multicollinearity, and therefore the results of Model A cannot be used to support our hypothesis.

3.2. Results from linear regression model B

Results of the residual model (Model B) showed that in all the 4-, 5-, and 6-day experiments, *ME* was insignificant ($P > 0.05$), whereas *SE* was significant ($p < 0.001$) in the 6-day MDA assays and 5-day CARB assay, and was close to significance ($P = 0.06$) in the 4-day MDA experiment (Table 2). All the VIFs and condition indexes in Model C were smaller than 1.2 indicating no multicollinearity. All statistical model assumptions (normality, constant variance, linearity) were met via checking residual plots and scatterplots. Also, there were no outliers that heavily influenced the regression fit.

The results of the regression coefficients of Model B strongly supported the predictions (Table 2). First, the coefficient of *SE* was much larger than that of *ME*, i.e., $\beta_2 > \beta_1$, (>20-fold, 109-fold, and 80-fold in the 4-, 5-, and 6-day experiments, respectively), indicating that *SE* had the highest correlation with *Damage* after accounting for the other variables (i.e., *SE* was more influential than *ME*). Second, the ratio $\beta_1/\beta_2 = (1-\varepsilon)/\varepsilon$gave similar estimates for ε, 0.989, 0.991 and 0.962 and in 6-, 5-, and 4-day experiments, respectively, which are remarkably close to the predicted value 0.99. Third, and more importantly, the *P*-values of *ME* were 0.29, 0.1, and 0.52 for 6-, 5-, and 4-day experiments respectively, indicating its insignificant effect on *Damage* level, whereas the *P*-values of *SE* were smaller than 0.001 and 0.038 in the 6-day MDA and 5-day CARB experiments respectively, and smaller than 0.06 in the 4-day MDA experiments.

The lower and upper bound of the parameter E_m, 143 and 212 J/g, gave slightly different results in the second model (Model B). The statistical parameters, i.e., the *R*-, *P*-, and *t*-values, and coefficient of *ME* (β_1) kept the same as in Table 2. The coefficient of *SE* and the repair efficiency became smaller for the upper bound, and larger for the lower bound. For the upper bound of E_m (212 J/g), $\beta_2 = 25.89$ (6-day) and 7.89 (4-day) from the MDA assays, $\beta_2 = 0.0865$ from the 5-day CARB assays , and the repair efficiency $\varepsilon = 0.986$ (6-day), 0.988 (5-day), and 0.952 (4-day). For the lower bound of E_m (143 J/g), $\beta_2 = 38.22$ (6-day) and 11.70 (4-day) from the MDA assay, and $\beta_2 = 0.128$ for the 5-day CARB assay, and $\varepsilon = 0.990$ (6-day), 0.992 (5-day), and 0.967 (4-day).

Finally, we estimated the effects of measurement errors in *ME*. The standard deviation of the measurement error was 17% of the mean values. Using this percentage value, we obtained the size of the measurement error standard deviation (σ_u) of *ME* in the 6-day experiment, 338.3 J. The regression coefficient estimates after fitting Model B with measurement error in *ME* were very similar to the initial Model B (Table 2). Taking the 6-day experiment as an example, the repair efficiency, ε, estimated from the coefficients in Model B with the measurement error is 0.988, slightly smaller than the ones estimated without the measurement error, but still remarkably close to the predicted value, 0.99.

4. Discussion

The results of this study shed new light on the oxidative stress hypothesis of aging, which recently has been seriously questioned, and even considered dead by some authors (e.g., (Pérez et al., 2009)). As we explained in a previous publication (Hou and Amunugama, 2015), many questions against this hypothesis stem from a common misunderstanding that this hypothesis assumes proportional relationships between the rates of metabolism (oxygen consumption), production reactive oxygen species, and cellular damage and longevity. In fact, oxidative stress hypothesis of aging only assumes a direct link between cellular damage and lifespan. In Assumption 1 of our model, we assumed a proportional relationship between ROS production and oxygen consumption rate (metabolic rate). However, there exists a natural leak of proton across the mitochondrial inner membrane. The fraction of respiration that drives the proton-leak is not involved in ROS production. Thus, the proton leak may cause disproportionality between ROS production and metabolic rate (Brand, 2000). Moreover, due to a series of factors under certain conditions, even after taking consideration of proton-leak, the net ROS level may still not be proportional to non-proton-leak dependent oxygen consumption (Barja, 2007; Barja, 2013; Hulbert et al., 2007). Here we make three points to address this issue.

First, some evidence (Salin et al., 2015; Speakman et al., 2004) has shown that under certain conditions, ROS production can be disproportionally low for a given oxygen consumption rate, probably due to high proton leak. In the context of our model, the low-ROS production due to proton leak means a low damage coefficient, δ. (Assumption 1: ROS/damage production, $H = \delta \times B$. coefficient δ is low, if *H* is low for a given *B* due to proton leak.) Recalling that repair efficiency is $\varepsilon = \eta/\delta$, a lower δ means a higher ε. Now, it comes back to the main point of this paper: if the repair efficiency ε is high, then the

damage will be insensitive to metabolic rate, because the contribution of metabolic energy to damage is $(1-\varepsilon)\times B$(Eq. (1)), and when ε is high, no matter how B changes, this term is close to zero.

Second, the main purpose of our model is to disentangle the effects of growth and the metabolic rate that fuels growth. The oxygen consumption that drives the proton leak does not produce ATP (energy), so it is not entangled with growth, and it is not even included in our energy partition equation. The major prediction of our model is that, even after taking consideration of proton-leak, the variation in proton-leak-independent metabolic rate is still not the major cause of the variation in cellular damage, opposite of the conventional thinking. Non-constant percentage of respiration that drives mitochondrial proton leak can cause variation in metabolic rate, but not all the variations in metabolic rate can be attributed to the variation in proton-leak. A series of environmental and physiological factors can alter metabolic rate without changing the percentage of proton leak. In this study, the variation in metabolic rate is mainly induced by food supplies. So, we assume that in this study even if there is a variation in proton leak across individual larvae, the observed variation in metabolic rate induced by this factor is negligible, compared to that induced by food supply.

Third, and more importantly, even after taking consideration of proton-leak, the net ROS level may still not be proportional to the proton-leak independent respiration. One of the major reasons of the disproportionalities is that many environmental or genetic factors can induce reshuffling in energy budget, retard (or accelerate) biosynthesis, and channel extra energy to (or away from) ROS scavenging and damage repair (Hou and Amunugama, 2015). One typical example is the low ROS level under diet restriction. Our model suggests that the highly efficient repair mechanisms scavenge most of the free radicals produced by oxidative metabolism and the consequent cellular oxidative damage, if energy required for scavenging and repair is sufficient. As we discussed previously in (Hou, 2013), the diet restriction-induced low ROS level may be attributed to the enhanced energy supply to maintenance. While mildly reducing or having no effect on metabolic rate, diet restriction largely suppresses growth, and channels more energy from biosynthesis to maintenance (scavenging). This is exactly the main point of this paper—biosynthesis rate has a larger impact on ROS damage than metabolic rate does. Indeed, reduction of growth plays a very important role in diet restriction's effect on lifespan extension. Hou (2013) has analyzed the empirical data from >200 studies of food restriction on small rodents, and found that growth reduction explains 86% of the lifespan extension by diet restriction.

Below we discuss a few pieces of indirect empirical evidence that supports our model.

Recently Salin et al. (2015) have shown that within a population of brown trout of same age and under identical environment, individuals with higher mass-independent metabolic rate have lower levels of ROS (H_2O_2). Although the proton-leak cannot be excluded as a cause for this negative correlation, the authors suggested that "it is feasible that individuals with a lower H_2O_2 level may have allocated more resources towards antioxidant defences." Noticing the fact that body mass of the fish at the same age ranged from 5.05 to 13.95 g, it is possible that the large variation in biosynthesis (growth) caused variation in the effort of ROS scavenging. I.e., individuals with smaller body size at the same age had spent less energy on biosynthesis, allocated more energy to scavenge H_2O_2, and therefore had low H_2O_2 level despite high metabolic rate.

Another line of indirect evidence comes from the studies that experimentally elevated metabolic rate, but failed to change growth rate, and had no harmful effect on health or lifespan. For example, Selman et al. (2008) exposed voles to lifelong coldness, which elevated their metabolic rate by almost 100%, but had minor effect on growth rate. The cold exposed group and the control reached the same body mass at 20-month old. The authors found "no treatment effect on cumulative mortality risk" and negligible effects on DNA oxidative damage, lipid peroxidation, and antioxidant protection. Similarly, moderate exercises increase energy expenditure, but have no effect lifespan (Holloszy, 1997), or in some cases even increase lifespan (Holloszy, 1993; Navarro et al., 2004). In our Eq. (1), $D=(1-\varepsilon)\times ME+\varepsilon\times SE$, the changes in damage induced by a large increase in metabolic energy (ME) can be offset by a slight decrease in SE (growth), because the coefficient of ME is much smaller than that of SE, i.e., $1-\varepsilon<<\varepsilon$.

However, in some studies of cold exposure or mild exercise, growth seems unchanged, while ME largely increases. So, in these cases our Eq. (1) predicts a net increase in damage, which contradicts with the empirical results. We suggest that the key to understanding the contradiction still lies in the high efficiency. When animals are under stress, some repair and protection-related gene expression can be up-regulated, a phenomenon known as hormesis (Masoro, 2005; Rattan, 2004; Ristow and Zarse, 2010). It has been hypothesized that cold exposure and mild exercise can induce such a hormetic effect (Rattan, 2004). The effect may alter the structure of the macro-molecules and make them more resistant to oxidative insults. In the context of our model, this means that the coefficient, δ, (the amount of mass caused by one unit of metabolic energy), is reduced by the hormetic effects. Moreover, during exercises the mitochondrial ROS production rate becomes lower when mitochondria transits from resting respiring state 4 to state 3 (the active phosphorylating respiration) (Barja, 2007), and this transition will also reduce δ. The mild stresses may also enhance the efficiency of repair or ROS scavenging, and increase the value of η. Recalling that the repair efficiency $\varepsilon=\eta/(\delta f)$, the reduced δ and increased η will increase ε.

The increase in ε induced by mild stress does not have to be large to offset the effect of increased metabolic rate. Here we give an approximate estimate to show this point. Using the physiological data of a typical rat as an example ($B_{rrest}(\text{watts})=3.4\times m^{3/4}$, $M=500$ g (Peters, 1983)), the total resting metabolic energy spent by a rat from birth to the age of 200 days is about $\int_0^{200\ d} B_0 m(t)^{3/4} dt \approx 34000$ Kilojoules. The energy spent on bio-tissue synthesis from birth (~5 g) to the age of 200 days is about 3000 Kilojoules(Moses et al., 2008). Taking the value of $\varepsilon=0.998$ for rat previously estimated in (Hou, 2013), the damage calculated by Eq. (1) is about $(1-\varepsilon)\times ME+\varepsilon\times SE\approx 3060$KJ. Now, we assume that under mild stress, metabolic energy, ME increases 100%, from 34,000 to 68,000 KJ, while SE keeps unchanged. It is straightforward to see from Eq. (1) that an increase in ε from 0.998 to 0.999 is sufficient to offset the large increase in ME, and keep the damage level unchanged.

We need to emphasize that efficiency ε is high and robust as the result of natural selection (Sohal et al., 2002). Not all the low dose stresses can induce hormetic effects and further increase it (Masoro, 2005). However, growth rate, on the other hand, is much more plastic. A series of environmental factors, such as food supply, can change it, and therefore change the damage in Eq. (1). Thus, many interventions, such as food restriction, extend lifespan by changing growth rate and inducing energy reshuffling between biosynthesis and maintenance.

During growth, both metabolic and biosynthetic rate vary constantly. A variety of genetic, environmental and physiological factors may cause independent or even opposite changes in these two rates. Since metabolic and biosynthetic rate may vary independently, their impacts on cellular damage may be different too. However, most studies on oxidative damage have only investigated the collective results of the concerted effects of these two rates. Our study offers a departure point for better understandings of their relative effects on cellular damage. This study also provides a theoretical framework for estimating how genetic, environmental, and physiological factors influence children's health during growth.

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Appendix A. Supplementary data

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